PHOSPHATE SOLUBILIZING MICROBES FROM CEREAL CROPS AND THEIR BENEFICIAL APPLICATIONS IN PLANT GROWTH UNDER RAINFED CONDITIONS

SYNOPSIS

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By

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SYNOPSIS

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I. INTRODUCTION

Sustainable agriculture plays an important role in today's world as it offers the potential to meet all our future agricultural needs without compromising the soil quality, something that conventional agriculture may not be able to do and it has basically evolved from mainly three perspectives: system of production so that self sufficiency in food can be achieved, concept of stewardship as well as the means by which the rural communities can be sustained. The principal goal of nature farming is that abundant and healthy crops can be produced without the use of the chemical fertilizers and pesticides, thereby minimizing adverse effect on the natural environment. The pesticides causes the environmental pollution, leads to the loss of the microbial diversity in the natural environment further making plants more susceptible to the pests and diseases. One of the most promising way of enhancing the crop productivity as well as managing the plant diseases is the use of the bio based pesticides and fertilizers such as use of effective micro organisms. Micro organisms are an unlimited source of the various novel substances with therapeutic applications and further, their secondary metabolite screening for pharmaceutically significant novel antibiotic and non antibiotic compounds as well as drug lead molecules has either assumed greater attention in recent times. Many soil inhabiting bacteria are known to produce the secondary metabolites which can suppress the micro organisms competing for the same resources (Garbeva et al. 2011). Use of plant growth promoting microbes (PGPM) plays an important role in the developing the promising method of crop management (Sturz et al. 2000a). Plant growth in the agricultural soils is influenced by various

biotic and abiotic factors. There is a thin layer of soil immediately surrounding the roots of plants which is extremely important as well as the active area for the root activity and metabolism and is referred to as the rhizosphere. Micro organisms that colonize the rhizosphere can be classified according to the effects they possess on the plants and the way they interact with the roots. Rhizobacteria inhabit the roots of plants and further exert a positive effect which ranges from direct influence mechanisms to indirect effects. Thus, the microbes which inhabit the roots and are beneficial to the plants are referred to as plant growth promoting rhizobacteria (PGPR). The plant growth promoting mechanisms by PGPR are of diverse nature such as the phosphorus solubilization, nitrogen fixation, production of the plant hormones (Bent et al. 2001; Reyes et al. 2002) such as auxins, gibberellins, cytokinins and excretion of various antibiotics or proteolytic enzymes. Some plant beneficial micro organisms antagonize plant pathogens through the competition for nutrients, parasitism by the production of the hydrolytic enzymes, inhibition of the plant pathogens by the production of anti- microbial compounds, induction of the systemic resistance in the host plants (Compant et al. 2005; Whipps 2001b). Interactions between bacteria and plants occur through endophytic, symbiotic or associative processes with the distinct degree of proximity with the roots and the surrounding soil. There are number of plant growth promoting microbial strains which are commercially used as the adjuncts to agricultural practice. These strains include *Agrobacterium*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Paenibacillus*, *Pseudomonas*, *Serratia* and *Streptomyces* (Bent et al. 2001; Compant et al. 2005; Raaijmakers et al. 2011; Reyes et al. 2002; Sturz et al. 2000b; Whipps 2001a).

One of the best studied mechanisms of plant growth promotion includes providing the plants with the nutrients that they lack such as the fixed nitrogen, iron and phosphorus. Phosphorus is one of the important components of the macromolecules which is present in the living systems and is important for the fertility of soil as well as the growth of the plants. The amount of the phosphate is quite high in the soil varying between 400 and 1200 mg Kg^{-1} of the soil, but most of it is present in the insoluble form and not available for the growth of the plants. Thus, the limited bioavailability of the phosphorus from the soil combined with the fact that this element is essential for the growth of the plants means the inability to obtain it in sufficient amounts limits the growth of the plant (Feng et al. 2004). The insoluble phosphorus is present either an inorganic mineral such as the apatite or as one of several organic forms such as the

inositol phosphate (soil phytate), phosphomonoesters and phosphotriesters (Khan et al. 2007). A large proportion of the supplied phosphorus gets fixed in the soil as the phosphates of iron, aluminium and calcium (Altomare et al. 1999) which further leads to the need of the frequent application of the phosphate fertilizers on regular basis which has become costly as well as environmentally undesirable and hence there is need for the alternative sources (Reddy et al. 2002). Phosphate solubilizing bacteria are well known to promote plant growth as they possess the ability to convert an insoluble form of phosphorus to soluble form by different processes such as the acidification, chelation and exchange reactions that is then readily taken up by the plant roots.

Another important role of PGPR is that they are the elicitors of the tolerance to the abiotic stresses such as the drought, salt, nutrient deficiency or excess (Arshad et al. 2008; Zahir et al. 2009). The scarcity of the water in the root zone for a longer period of time which hampers the growth of the plant is known as the drought and it imposes serious reduction in the crop yield and is one of the greatest limitations faced by the present day agriculture. PGPR posses various properties like exopolysaccharide (EPS) production and 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme activity to cope up with the various biotic and abiotic stresses such as salt stress, flooding stress, drought stress, heavy metal stress. The EPS envelope basically protects bacteria from the drought by enhancing water retention and also from the fluctuations in water potential by regulating the diffusion of the organic carbon sources (Yilmaz et al. 2012).
Looking at the importance of P-solubilizing rhizospheric microbes in enhancing the crop growth of different crops by P-solubilization and others plant growth promoting attributes, the present investigation would be carried out with the following objectives:

1. Isolation and characterization of rhizospheric microbes from cereal crops
2. *In vitro* characterization of microbes for drought tolerance, P-solubilization and other plant growth promoting attributes
3. Molecular characterization of selected drought tolerant P-solubilizing microbes
4. Applications of drought tolerant P-solubilizing microbes for enhanced plant growth and P-uptake under drought conditions

THE RATIONALE OF THE PROPOSED STUDY

Phosphate (P) is the major essential macronutrients for biological growth and development. However, the concentrations of soluble P in soil are usually very low, as the biggest proportions of P in soil are insoluble rocks, minerals and other deposits. It is important for the plant growth and promotes root development, tillering and early flowering and performs several other functions like metabolic activities, particularly in synthesis of protein and nucleic acid. The concentrations of soluble P in soil are usually very low, as the biggest proportions of P in soil are insoluble rocks, minerals and other deposits. It is one of the major growth-limiting nutrients in plants and is often the limiting mineral nutrient for biomass production in natural ecosystems. It is important for the plant growth and promotes root development, tillering and early flowering and performs other functions like metabolic activities, particularly in synthesis of protein and nucleic acid. Phosphates applied to agricultural soils in the form of chemical fertilizers are rapidly immobilized and rendered inaccessible for plants. Due to this rapid immobilization, many agricultural soils have large reservoirs of phosphates in inaccessible forms. Furthermore, excessive exploitation of phosphate reservoirs for the synthesis of the chemical fertilizers has led to the threat of their complete depletion in next 10-15 years. Therefore, the identification and utilization of efficient P-solubilizing microbial isolates with other plant growth promoting attributes will not only provide an alternate to chemical P-fertilizers but also enrich soil and plant health as an eco friendly approach. Owing to the importance of the P-solubilizing microbes towards the potential application for sustainable agriculture and plant growth promotion, numerous studies have conducted on these microbes from different sources and environmental conditions.

II. REVIEW OF LITERATURE

Soil is dynamic, living matrix which is a very essential part of terrestrial ecosystem. It is a very critical resource for the agricultural production, food security and is also important for maintenance of most of the life processes. The functions of the soil biota are central to the decomposition processes and nutrient cycling. There are several minerals which consist of essential elements, the most important being nitrogen, phosphorus and potassium. Soil is considered to be the store house of the microbial activity though the space which is occupied by the micro organisms is estimated to be less than 5% of the total space and the major microbial activity is confined to the hotspot which basically include the aggregates with the accumulated organic matter and the rhizosphere. Micro organisms are involved in basic ecosystem processes such as the biogeochemical cycles, food chains, further they also maintain a required relationships between themselves and the higher organisms. Further, the soil micro organisms also play a vital role in the soil ecosystem, its functioning related to the soil fertility and decomposition of the organic matter. Inspite of being bestowed with a remarkable inherent physiological and functional diversity, micro organisms further also find applications in the fields of agriculture, industry, medicine and environment.

The microbial diversity of soil is higher than any other microbial communities. Thus, it becomes very important to explore, preserve, conserve and utilize unique microbial flora to fulfill the emerging needs of the society, industries and clean environment. In conventional agriculture, chemical fertilizers, pesticides and the plant growth regulators are applied so that the yield as well as the quality of crops could be increased. However, the frequent and excessive use of these chemicals results in the adverse environmental effects, cause disturbances in the ecological balance of the soils and ultimately making the plants more susceptible to pests and diseases. Soil is replete with bacteria, fungi, algae, actinomycetes and protozoa and of these; bacteria and fungi are by far the most abundant. Plants interact with the broad range of bacteria and fungi that colonize rhizosphere (rhizobacteria), phyllosphere (epiphytes), inside of plant tissues (endophytes). The number and type of bacteria in the soil depends on the soil conditions such as the temperature, moisture, presence of salt and other chemicals as well as the number and type of the plants found in those soils. Additionally, microbes are not evenly distributed in the soil and the concentration of microbes around the roots of the plants *i.e,* in the rhizosphere is much greater than rest of the soil due to the presence of the nutrients such as amino acids, sugars,

organic acids and other small molecules from plant root exudates. Many members of rhizospheric microbiome are beneficial to the plant growth even the microorganisms which are pathogenic to the plants are also present. The plant-microbe interactions in the rhizosphere are basically the determinants of plant health, productivity as well as the fertility of the soil. Rhizospheric organisms which have been well studied for their beneficial effects on plant growth and health mainly includes nitrogen fixing bacteria, mycorrhizal fungi, and biocontrol micro-organisms.

Environmentally friendly biotechnological approaches offer alternatives to the chemical fertilizers. The negative environmental impacts of the chemical fertilizers and their increasing costs, the use of PGPM is thus considered to be the supplemental way of reducing the use of chemicals in the agriculture. It has been estimated that in the soil upto about 75% of the applied phosphate fertilizers become unavailable to the plants because of the mineral phase re-precipitation. Microbial solubilization of P and its use in agriculture is receiving a greater attention now a day. The phosphate solubilizing microbes (PSM) could play an important role in supplying P to the plants in a sustainable and eco-friendly manner with the optimum dosage of P fertilizer. The PSM are able to convert insoluble phosphates into the soluble forms and this phosphate solubilizing microbes have therefore been used so as to enhance the solubilization of the re-precipitated phosphorus for the crop improvement. Phosphate solubilizing microbes have been isolated from various environments including the agro-industrial wastes (Vassileva et al. 2010) sub tropical soil (Chen et al. 2006), rice and banana fields (Naik et al. 2008), rhizosphere soil of sugarcane plant (Upadhyay et al. 2009), maize (Vyas and Gulati 2009). In a study by Cattelan et al. (1999) isolates from the rhizosphere of soyabean were found to solubilize P *in vitro* along with other plant growth promoting traits and increased the growth of soyabean. The most efficient phosphate solubilizing microbes belong to the following genera *Bacillus, Rhizobium* and *Pseudomonas*. *Pseudomonas* and *Azospirillum* have been isolated from the rhizospheric soil and root cuttings of *Piper nigrum* L exhibited high phosphate solubilizing ability *in vitro* (Ramachandran et al. 2007).

Raj et al. (2014), isolated and molecularly characterized phosphate solubilizing rhizobacteria from pristine soils and the dominant PSM found were *Bacillus thuringiensis, Pseudomonas panipatensis, Sinomonas atrocyanea* and *Pseudomonas monteilii*. Midekssa et al. (2015) isolated and characterized phosphate solubilizing rhizobacteria from lentil growing areas

of Ethiopia, *Bacillus* (10%), *Enterobacter* (10%), *Chrysomonas* (5%), *Sphingomonas* (2.5%) and *Agrobacterium* (2.5%) were found to be the dominant genera. There are even many studies which have reported that there is increase in the plant biomass when treated with the PSM. Elkoca et al. (2007) demonstrated that there was increase in the plant height as well as root and shoot dry weights in *Cicer arietium* (chick pea) when it was inoculated with the *Bacillus megaterium* in a field trial. The inoculation of maize with *Serratia marcescens* and *Pseudomonas* sp. showed increase in the biomass by 50% and 18% respectively in 96 day field trial (Hameeda et al. 2008). Seed inoculation of *Elusine coracana* (finger millet), *Zea mays* (maize), *Amaranthus hypochondriacus* (amaranth), *Fagopyrium esculentum* (buckwheat), *Phaselous vulgaris* (French bean) with *Bacillus* sp. isolates from pasture and wetland were found to increase crop production.

Mineral forms of phosphorus are represented in the soil by primary minerals which include apatite, hydroxyapatite and oxyapatite. They are found as the part of stratum rock and their main characteristic feature is their insolubility. One of the major reason of unavailability of the phosphorus for the plants is its reactivity with various metal complexes such as Fe, Al and Ca which leads to the precipitation or adsorption of between 75-90% of the P in the soil (Gyaneshwar et al. 2002; Igual et al. 2001). These insoluble, precipitated forms cannot be absorbed by plants and only about 0.1% of the total P exists in a soluble form which is available for uptake by the plants because of its fixation into an unavailable form due to P fixation. There are mainly two forms of P in the soil organic and inorganic phosphates. Organic matter is an important reservoir of immobilized P that accounts for about 20-80% of the soil P. This organic P is largely present in the form of inositol phosphate (soil phytate) and is the most stable form of P accounting for about 50% of the total organic phosphorus and is synthesized by microbes as well as plants.

The organic form of the phosphorus cannot be taken up by the plants directly and it has to be transformed into the inorganic form after being mineralized and catalyzed by different soil enzymatic processes (Sarapatka 2003). The phosphatases in the soil basically cause the hydrolysis of the soil organic phosphorus and convert it into the inorganic forms such as the HPO_4^{-} and $H_2PO_4^{-}$ before it can be utilized and taken up by the plant roots from the soil solution. Phosphatases basically fall under categories a) Non-specific acid phosphatases which performs the dephosphorylation of the phosphor-ester or phospho-anhydride bonds present in the organic

matter and these predominate in the acidic soil b) Phytases, most of them belonging to high molecular weight acid phosphatases. They specifically cause the release of the P through phytate degradation. c) Phosphonatases and C-P lyases, which performs the C-P cleavage in organophosphonates. Inorganic P occurs in the form of insoluble mineral complexes and some of these occur after the application of chemical fertilizers whereas organic forms of P include phospholipids, nucleotides and inositol phosphate. The organic phosphorus is an important reservoir of immobilized and accounts for about 20-80% of the total soil P. The organic phosphorus of soil plays an important role in the nutrition of the crops especially in the high P-fixing calcareous soils. Thus, high Pi fertilizers are applied so as to meet the requirements of the crops which not only add major cost to the agricultural production but over application of these chemical fertilizers can result in the negative effects such as leaching, pollution of water resources, destruction of micro organisms and friendly insects.

Inorganic P in acidic soils basically forms a complex with Fe and Al compounds whereas in neutral or calcareous soils calcium phosphate predominates. There are wide range of mechanisms which exist in nature for P solubilization by microbes and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi. Inorganic P which is present in the soil is solubilized by the action of various organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, and Ca) and also known to decrease the pH in basic soils. Gluconic acid is the major acid released by PSM in the solubilization of phosphate. There are other acids also which are released including citric acid, lactic acid, oxalic acid, tartaric acid. fumaric, glycolic, malonic and succinic acids. There are various reports which suggest the ability of the bacterial species to solubilize inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate and make it available to the plants. These include *Achromobacter*, *Aerobacter*, *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Erwinia*. *Flavobacterium, Micrococcus, Pseudomonas, Rhizobium* and *Serratia*.

The genetic basis of phosphate solubilization is still not well understood. The production of the organic acids due to the chelation of calcium ions from mineral P precipitates to the anion provided in organic acids is considered to be the basic mechanism for mineral phosphate solubilization. Acid production basically elevates positive ions in the periplasmic space of PSM reducing the surrounding pH in the associated rhizosphere. The production of the gluconic acid

via direct oxidation pathway by PSB has been identified to be an important mechanism for mineral phosphate solubilization. Chen et al. (2006), reported four strains namely *Arthrobacter ureafaciens*, *Phyllobacter myrsinacearum*, *Rhodococcus erythropolis*, *Delftia* sp. as phosphate solubilizing bacteria after confirming their capacity to solubilize considerable amounts of tricalcium phosphate in the medium by secreting organic acids. Glucose dehydrogenase (Gcd) is the member of largest group of quinoproteins which are located in the periplasmic space of cytoplasmic membrane of Gram negative bacteria (de Werra et al. 2009). Gcd protein requires redox co factor pyrroloquinoline quinine (PQQ) and the biosynthesis of PQQ requires six genes (pqq A-F) encoded by an operon that varies among the bacterial strains.

Goldstein and Liu (1987), firstly cloned a gene from *Erwinia herbicola* which is involved in the phosphate solubilization by screening the antibiotic recombinants from a genomic library in a medium which consisted of hydroxyapatite as a source of P. Then, the expression of the gene in the strain of *E. coli* HB101 allowed the production of the gluconic acid and mineral phosphate solubilization activity. Further, sequence analysis of this gene (Liu et al. 1992) suggested its probable involvement in the synthesis of enzyme PQQ synthase which directs the synthesis of PQQ which is a co-factor necessary for the formation of the holoenzyme glucose dehydrogenase (GDH-PQQ) which basically catalyzes the formation of gluconic acid from glucose by oxidation pathways. Among rhizobacteria, a gene that facilitates phosphatase activity was isolated from *Burkholderia cepacia* (Rodríguez et al. 2000). This gene basically codes for an outer membrane protein that enhances the synthesis in the absence of soluble phosphates in the medium, and could be involved in P transport to the cell. Besides, cloning of other two nonspecific periplasmic acid phosphatase genes i.e, napD and napE from *Rhizobium* (*Sinorhizobium*) *meliloti* was accomplished (Deng et al. 1998) (Deng et al. 2001).

In a study of Buch et al. (2010) phosphoenolpyruvate carboxylase (*ppc*) from *Synechococcus elongates* was overexpressed in *Pseudomonas fluorescens* under a P limiting environment. As a result, phosphoenolpyruvate carboxylase production increased by12-fold, and carbon flow decreased via the direct oxidative pathway. Overexpression of *ppc* was found to reduce Gcd activity and increased associated glucose-6-phosphate dehydrogenase activity. This was correlated with increased release of pyruvate and acetate which are the final products of intracellular phosphorylative oxidation of glucose. Patching the bacteria on dicalcium phosphate Pikovskaya's agar there was increase in the zone of clearance in the mutant that carried the *pcc*

gene relative to wildtype. It was suggested that the factors involved in 6-phosphogluconate pathway and the subsequent tricarboxylic acid cycle in *P. fluorescens* could provide an attractive new pathway for genetic engineering strategies to increase the efficiency of mineral phosphate solubilization by PSB rather than targeting PQQ biosynthesis.

Another important role of PGPM is in enhancing the adaptation of the plants to overcome abiotic stress such as drought, salinity in soil or too cold or too hot temperature and represents the most economic problem for the crop production worldwide. PGPM are able to increase the plant tolerance *via* different mechanisms including the lowering of the ethylene concentration in plants, producing phytohormones, regulating nutrient uptake, inducing and augmenting stress response gene expression or production of the antioxidants. Drought is one of the major environmental stresses which limit the plant growth and consequently the crop yield. It has been estimated that about 45% of the land is subjected to continuous or frequent drought conditions. Drought is defined as the situation that basically lowers the water potential and turgor to the extent that plant faces difficulties in executing normal physiological functions. The phytohormone ethylene basically modulates plant growth and development as well as plant response to abiotic stress and is one of the most widely used hormones in the agriculture to increase yield and production costs. Studies have shown that inhibitors of ethylene biosynthesis and perception can mitigate yield loss by enhancing the plant tolerance to abiotic stress (Hays et al. 2007; Huberman et al. 2014). Ethylene being a plant growth hormone is also referred as the stress hormone (Saleem et al. 2007). Whenever there is stress conditions the endogenous level of ethylene is significantly increased which negatively affects the growth of the plant. For instance, the high concentration of ethylene induces defoliation and other cellular processes which ultimately reduces the crop performance (Bhattacharyya and Jha 2012; Saleem et al. 2007). Research is being conducted for the mechanisms behind the plant stress tolerance enhancement in order to find the possibilities for economically efficient rhizobacterial applications for plant drought tolerance enhancement. The very first report on the enhancement of the drought tolerance by the PGPR was published in Sweden, it was shown that *Arabidopsis thaliana* inoculated with PGPR *Paenabacillus polymyxa* B2 could survive a longer period of drought in comparison to the untreated control plants.

PGPM basically possess an enzyme ACC deaminase which basically decreases the levels of ethylene thereby reducing drought stress in plants. ACC breakdown and ethylene

synthesis inhibition by ACC deaminase decreases the damage of various stress situations by enhancing homeostasis in and around the plant root, especially at the early stages of stress exposure and thus the ACC deaminase can be considered as one of the signaling compounds mediating the plant basic stress tolerance. The presence of ACC deaminase has been reported in all three genera i.e, eukarya, archea as well as the bacteria but the ACC deaminase activity has been known to be present majorily in the bacteria and fungi. The ACC deaminase activity has also been reported in a hyperthermophilic archaeon, *Pyrococcus horikoshii* (Fujino et al. 2004). Among eukaryotes production of the ACC deaminase has been reported in some fungi which includes few species of yeasts including *Hansenula saturnus* (Minami et al. 1998) *Issatchenkia occidentalis* (Palmer et al. 2007). The structural gene *Acd S* which encodes ACC deaminase has been confirmed at the molecular level by amplification and sequence analysis. The gene has been found in the *Actinobacteria, Deinococcus-Thermus,* three classes of Proteobacteria (α, β, and γ), in fungi belonging to Ascomycota and Basidiomycota. The presence of ACC deaminase activity has also been reported in bacteria belonging to phyla Chlorobi, Bacteroidetes and Firmicutes (Nascimento et al. 2014). Recently, the bacterial strains exhibiting ACC activity has been identified in the wide range of the genera such as *Acinetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia*, *Rhizobium* (Kang et al. 2010; Nadeem et al. 2009; Zahir et al. 2008).

Another important characteristic feature of PGPM is that they possess exopolysaccharide production which also helps to cope up with the variety of the abiotic stress and biotic stress. Generally, the microorganisms live within the envelope of EPS matrix in various environments and soil being one of them. This EPS matrix protects bacteria from drought by enhancing water retention and from fluctuations in water potential by regulating the diffusion of the organic carbon sources. Further, EPS also helps the bacteria in colonization of the plant roots due to fibrous network material that connects bacteria to root surface.

III. METHODOLOGY

1. **Isolation and characterization of rhizospheric microbes from cereal crops**
    - Survey and collection of samples from cereal crops growing in Sirmour district, Himachal Pradesh.
    - Isolation of rhizospheric microbes using different growth media such as: Nutrient agar medium, King's B agar, T_3 agar, Tryptic soy agar, Potato dextrose agar, Rose bengal agar etc.
    - Cultural characterization of isolated microbes
2. ***In vitro* characterization of microbes for drought tolerance, P-solubilization and other plant growth promoting attributes**
    - *In vitro* characterization of microbes for drought tolerance using polyethylene glycol (PEG-8000)
    - Screening of selected drought tolerant microbes for P-solubilization attributes using different insoluble P-sources
    - Screening of P- solubilizing microbes for other plant growth promoting attributes (solubilization of K and Zn; production of phytohormones and siderophores and hydrolytic enzymes.
3. **Molecular characterization of selected drought tolerant P-solubilizing microbes**
    - Isolation of genomic DNA from selected microbes
    - PCR amplification of 16S rDNA, ITS and candidate drought tolerant gene (s)
    - Sequencing of 16S rRNA and ITS gene for identification of bacteria and fungi respectively
    - Phylogenetic analyses using 16S rRNA and ITS genes using MEGA 6
    - Sequencing of candidate drought tolerant gene (s) and its BLAST analysis
4. **Applications of drought tolerant P-solubilizing microbes for enhanced plant growth and P-uptake under drought conditions**
    - Seed germination bioassay of selected microbial isolates with P-solubilizing and other PGP attributes under normal and drought conditions
    - Evaluation of rhizospheric drought tolerant P-solubilizing microbes for plant growth and P-uptake

IV. PLAN OF RESEARCH WORK

Time schedule of activities and activity milestones

S. No.	Objectives	When to be attained					
		1st Year		2nd Year		3rd Year	
		I	II	I	II	I	II
1.	Isolation and characterization of rhizospheric microbes from cereal crops						
2.	*In vitro* characterization of microbes for drought tolerance, P-solubilization and other plant growth promoting attributes						
3.	Molecular characterization of selected drought tolerant P-solubilizing microbes						
4.	Applications of drought tolerant P-solubilizing microbes for enhanced plant growth and P-uptake under drought conditions						

V. WORK DONE

Objective 1: Isolation and characterization of rhizospheric microbes from cereal crops

- Plant samples from cereal crops [maize, millet amaranth and buckwheat) were collected from Baru sahib, Sirmaur, Himachal Pradesh, India.
- Rhizospheric microbes has been isolated using eleven different selective and complex growth media
- A total of 180 bacterial and 13 fungal isolates have been obtained from maize, millet, *Amaranthus* and buckwheat.
- Isolated microbes have been characterized for their morphological and cultural characteristics

Objective 2: *In vitro* characterization of microbes for drought tolerance, P-solubilization and other plant growth promoting attributes

- All the 193 isolates have been screened for drought tolerance using PEG-8000 infused agar plates
- 130 bacterial isolates and 7 fungal isolates were found to drought tolerant upto 5% PEG-8000
- The drought tolerant microbes were further screened for P-solubilization and it was found that 12 bacterial isolates (1981.63 to 6679.79 $\mu g.L^{-1}$) and 3 fungal isolates (923.88 to 2031.49 $\mu g.L^{-1}$) were potential p-solubilizers
- The potential drought tolerant P-solubilizing microbes have been screened for other plant growth promoting attributes such as zinc and potassium solubilization, production of siderophores, ammonia, HCN, hydrolytic enzymes.

VI. EXPECTED OUTCOMES OF RESEARCH

- Advancement in taxonomy of rhizospheric microbes of cereal crops
- Identification of novel species/strain of P-solubilizing rhizospheric microbes
- Identification of microbes with P-solubilizing and drought stress traits
- Development of P-solubilizing bio- inoculants for cereal crops growing under rainfed conditions

REFERENCES

Altomare C, Norvell W, Björkman T, Harman G (1999) Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum Rifai* 1295-22. Applied and Environmental Microbiology 65:2926-2933

Arshad M, Shaharoona B, Mahmood T (2008) Inoculation with *Pseudomonas* spp. containing ACC-deaminase partially eliminates the effects of drought stress on growth, yield, and ripening of pea (*Pisum sativum* L.). Pedosphere 18:611-620

Bent E, Tuzun S, Chanway CP, Enebak S (2001) Alterations in plant growth and in root hormone levels of lodgepole pines inoculated with rhizobacteria. Canadian Journal of Microbiology 47:793-800

Bhattacharyya P, Jha D (2012) Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World Journal of Microbiology and Biotechnology 28:1327-1350

Buch A, Archana G, Kumar GN (2010) Heterologous expression of phosphoenolpyruvate carboxylase enhances the phosphate solubilizing ability of fluorescent pseudomonads by altering the glucose catabolism to improve biomass yield. Bioresource Technology 101:679-687

Cattelan A, Hartel P, Fuhrmann J (1999) Screening for plant growth–promoting rhizobacteria to promote early soybean growth Soil Science Society of America Journal 63:1670-1680

Chen Y, Rekha P, Arun A, Shen F, Lai W-A, Young C (2006) Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities Applied soil ecology 34:33-41

Compant S, Duffy B, Nowak J, Clément C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects Applied and environmental microbiology 71:4951-4959

de Werra P, Péchy-Tarr M, Keel C, Maurhofer M (2009) Role of gluconic acid production in the regulation of biocontrol traits of Pseudomonas fluorescens CHA0 Applied and environmental microbiology 75:4162-4174

Deng S, Elkins JG, Da LH, Botero LM, McDermott TR (2001) Cloning and characterization of a second acid phosphatase from Sinorhizobium meliloti strain 104A14 Archives of microbiology 176:255-263

Deng S, Summers ML, Khan M, McDermott TR (1998) Cloning and characterization of a Rhizobium meliloti nonspecific acid phosphatase Archives of microbiology 170:18-26

Elkoca E, Kantar F, Sahin F (2007) Influence of nitrogen fixing and phosphorus solubilizing bacteria on the nodulation, plant growth, and yield of chickpea Journal of Plant Nutrition 31:157-171

Feng K, Lu H, Sheng H, Wang X, Mao J (2004) Effect of organic ligands on biological availability of inorganic phosphorus in soils Pedosphere 14:85-92

Fujino A, Ose T, Yao M, Tokiwano T, Honma M, Watanabe N, Tanaka I (2004) Structural and enzymatic properties of 1-aminocyclopropane-1-carboxylate deaminase homologue from Pyrococcus horikoshii Journal of molecular biology 341:999-1013

Garbeva P, Silby MW, Raaijmakers JM, Levy SB, de Boer W (2011) Transcriptional and antagonistic responses of Pseudomonas fluorescens Pf0-1 to phylogenetically different bacterial competitors The ISME journal 5:973-985

Goldstein A, Liu S (1987) Molecular cloning and regulation of a mineral phosphate solubilizing gene from Erwinia herbicola Nature Biotechnology 5:72-74

Gyaneshwar P, Naresh Kumar G, Parekh L, Poole P (2002) Role of soil microorganisms in improving P nutrition of plants Plant and soil 245:83-93

Hameeda B, Harini G, Rupela O, Wani S, Reddy G (2008) Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna Microbiological research 163:234-242

Hays DB, Do JH, Mason RE, Morgan G, Finlayson SA (2007) Heat stress induced ethylene production in developing wheat grains induces kernel abortion and increased maturation in a susceptible cultivar Plant Science 172:1113-1123

Huberman M, Riov J, Goldschmidt EE, Apelbaum A, Goren R (2014) The novel ethylene antagonist, 3-cyclopropyl-1-enyl-propanoic acid sodium salt (CPAS), increases grain yield in wheat by delaying leaf senescence Plant Growth Regulation 73:249-255

Igual J, Valverde A, Cervantes E, Velázquez E (2001) Phosphate-solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study Agronomie 21:561-568

Kang BG, Kim WT, Yun HS, Chang SC (2010) Use of plant growth-promoting rhizobacteria to control stress responses of plant roots Plant Biotechnology Reports 4:179-183

Khan MS, Zaidi A, Wani PA (2007) Role of phosphate-solubilizing microorganisms in sustainable agriculture—a review Agronomy for sustainable development 27:29-43

Liu S-t et al. (1992) Cloning of an Erwinia herbicola gene necessary for gluconic acid production and enhanced mineral phosphate solubilization in Escherichia coli HB101: nucleotide sequence and probable involvement in biosynthesis of the coenzyme pyrroloquinoline quinone Journal of Bacteriology 174:5814-5819

Midekssa MJ, Loscher CR, Schmitz RA, Assefa F (2015) Characterization of phosphate solubilizing rhizobacteria isolated from lentil growing areas of Ethiopia African Journal of Microbiology Research 9:1637-1648

Minami R et al. (1998) Properties, sequence, and synthesis in Escherichia coli of 1-aminocyclopropane-l-carboxylate deaminase from Hansenula saturnus The Journal of Biochemistry 123:1112-1118

Nadeem SM, Zahir ZA, Naveed M, Arshad M (2009) Rhizobacteria containing ACC-deaminase confer salt tolerance in maize grown on salt-affected fields Canadian journal of microbiology 55:1302-1309

Naik PR, Raman G, Narayanan KB, Sakthivel N (2008) Assessment of genetic and functional diversity of phosphate solubilizing fluorescent pseudomonads isolated from rhizospheric soil BMC microbiology 8:1

Nascimento FX, Rossi MJ, Soares CR, McConkey BJ, Glick BR (2014) New insights into 1-aminocyclopropane-1-carboxylate (ACC) deaminase phylogeny, evolution and ecological significance PLoS One 9:e99168

Palmer C, Golden K, Danniels L, Ahmad H (2007) ACC deaminase from Issatchenkia occidentalis J Biol Sci 7:188-193

Raaijmakers JM, Silby MW, Garbeva P, Levy SB, De Boer W (2011) Transcriptional and antagonistic responses of Pseudomonas fluorescens Pf0-1 to phylogenetically different bacterial competitors The ISME journal 5:973

Raj D, Linda R, Babyson RS (2014) Molecular characterization of phosphate solubilizing bacteria (PSB) and plant growth promoting rhizobacteria (PGPR) from pristine soils Int J Innov Sci Eng Technol 1:317-324

Ramachandran K, Srinivasan V, Hamza S, Anandaraj M (2007) Phosphate solubilizing bacteria isolated from the rhizosphere soil and its growth promotion on black pepper (Piper nigrum L.) cuttings Plant Soil Sci 102:325-331

Reddy MS, Kumar S, Babita K, Reddy M (2002) Biosolubilization of poorly soluble rock phosphates by Aspergillus tubingensis and Aspergillus niger Bioresource Technology 84:187-189

Reyes I, Bernier L, Antoun H (2002) Rock phosphate solubilization and colonization of maize rhizosphere by wild and genetically modified strains of Penicillium rugulosum Microbial Ecology 44:39-48

Rodríguez H, Rossolini GM, Gonzalez T, Li J, Glick BR (2000) Isolation of a gene from Burkholderia cepacia IS-16 encoding a protein that facilitates phosphatase activity Current microbiology 40:362-366

Saleem M, Arshad M, Hussain S, Bhatti AS (2007) Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture Journal of industrial microbiology & biotechnology 34:635-648

Sarapatka B (2003) Phosphatase activities (ACP, ALP) in agroecosystem soils vol 396.

Sturz A, Christie B, Nowak J (2000a) Bacterial endophytes: potential role in developing sustainable systems of crop production Critical Reviews in Plant Sciences 19:1-30

Sturz AV, Christie BR, Nowak J (2000b) Bacterial endophytes: potential role in developing sustainable systems of crop production Critical reviews in plant sciences 19:1-30

Upadhyay SK, Singh DP, Saikia R (2009) Genetic diversity of plant growth promoting rhizobacteria isolated from rhizospheric soil of wheat under saline condition Current microbiology 59:489-496

Vassileva M, Serrano M, Bravo V, Jurado E, Nikolaeva I, Martos V, Vassilev N (2010) Multifunctional properties of phosphate-solubilizing microorganisms grown on agro-industrial wastes in fermentation and soil conditions Applied microbiology and biotechnology 85:1287-1299

Vyas P, Gulati A (2009) Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent Pseudomonas BMC microbiology 9:1

Whipps JM (2001a) Microbial interactions and biocontrol in the rhizosphere Journal of experimental Botany 52:487-511

Whipps JM (2001b) Microbial interactions and biocontrol in the rhizosphere Journal of experimental Botany 52:487-511

Yilmaz M, Celik GY, Aslim B, Onbasili D (2012) Influence of carbon sources on the production and characterization of the exopolysaccharide (EPS) by Bacillus sphaericus 7055 Strain Journal of Polymers and the Environment 20:152-156

Zahir Z, Munir A, Asghar H, Shaharoona B, Arshad M (2008) Effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (Pisum sativum) under drought conditions J Microbiol Biotechnol 18:958-963

Zahir ZA, Ghani U, Naveed M, Nadeem SM, Asghar HN (2009) Comparative effectiveness of Pseudomonas and Serratia sp. containing ACC-deaminase for improving growth and yield of wheat (Triticum aestivum L.) under salt-stressed conditions Archives of microbiology 191:415-424

ADVISORY COMMITTEE MEETING

Name of the student (Capital letters): **DIVJOT KOUR** Regd No.: **BS15PSBT006**
Major subject: **Biotechnology**
Major advisor: **Dr. Ajar Nath Yadav**
Department: **Biotechnology** College: **Akal College of Agriculture**

Venue: Department of Biotechnology, **Dated:** 07-12-2017 **Time:** 3.00 pm

Title of the thesis: Phosphate Solubilizing Microbes from Cereal Crops and their Beneficial Applications in Plant Growth under the Rainfed Conditions

Research work carried out

- Plant samples from cereal crops [maize, millet amaranth and buckwheat) were collected from Baru sahib, Sirmaur, Himachal Pradesh, India. Rhizospheric microbes have been isolated using eleven different selective and complex growth media.
- A total of 180 bacterial and 13 fungal isolates have been obtained from maize, millet, *Amaranthus* and buckwheat. Isolated microbes have been characterized for their morphological and cultural characteristics.
- All the 193 isolates have been screened for drought tolerance using PEG-8000 infused agar plates. 130 bacterial isolates and 7 fungal isolates were found to drought tolerant upto 5% PEG-8000
- The drought tolerant microbes were further screened for P-solubilization and it was found that 12 bacterial isolates (1981.63 to 6679.79 $\mu g.L^{-1}$) and 3 fungal isolates (923.88 to 2031.49 $\mu g.L^{-1}$) were potential p-solubilizers. The potential drought tolerant P-solubilizing microbes have been screened for other PGP attributes.

Research work to be carried out

- The microbes will be molecularly characterized for identification and other gene(s) responsible for drought tolerant
- Evaluation of drought tolerant P-solubilizing microbes for plant growth and P-uptake

Signature of the student

(Dr. Ajar Nath Yadav)
Signature of the major advisor

Committee Members

1. Dr. Vinod Kumar, Biotechnology.....
2. Dr. H.S. Dhaliwal, Agriculture.....
3. Dr. Sunil Kumar, Renewable Energy.....
4. Dr. N Ramu, Agronomy.....

ADVISORY COMMITTEE

Name of the Student: **Ms Divjot Kour**

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Department: **Biotechnology**

College: **Akal College of Agriculture**

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S. No.	Name and Department	Role	Signature
1.	Dr. Ajar Nath Yadav, Biotechnology	Major Advisor	
2.	Dr. Vinod Kumar, Biotechnology	Co-Major Advisor	
3.	Dr. H.S. Dhaliwal, Agriculture	Member	
4.	Dr. Sunil Kumar, Renewable Energy	Member	
5.	Dr. N Ramu, Agronomy	Member	

HOD
(Signature)

Major Advisor
(Signature)

Dean PGS
(Signature)

Synopsis approved as such
9/4

Dean of College
(Signature)

For Approval

Vice Chancellor